Phosphoproteome Changes in Altered Cholesterol Metabolism revealed by SCX Tip based Fractionation of Batch-enriched Phosphopeptides

Alireza Dehghani, Markus Gödderz, Volkmar Gieselmann and Dominic Winter

Institute for Biochemistry and Molecular Biology, University of Bonn, Germany; dominic.winter@uni-bonn.de

universitätbonn

OVERVIEW

The identification of large numbers of phosphopeptides requires enrichment from increased amounts of sample material due to the sub-stoichiometric appearance of phosphorylated peptides. The common approach to handle this increased amount is in solution digestion followed by peptide fractionation and phosphopeptide enrichment of the resulting single fractions. This approach is time-consuming and requires expensive chromatography systems / columns as well as experienced operators. This makes it difficult for unspecialized laboratories to perform such experiments. We present an approach employing fractionation of batch-enriched phosphopeptides by strong cation exchange (SCX) tips. We achieve superior results compared to samples prepared using the common strategy at a fraction of time and costs. We applied the workflow to the quantitative analysis of altered cholesterol metabolism in SILAC mouse embryonic fibroblasts by treatment with the cholesterol transport inhibitor U18666A.

INTRODUCTION

Phosphorylation is one of the most abundant and best studied posttranslational modifications in mammalian cells. To date, the majority of phosphorylation sites has been detected by untargeted large scale studies using specific enrichment for phosphorylated peptides. A common strategy in such experiments is the fractionation of large amounts of peptides followed by enrichment for phosphopeptides from the resulting fractions as phosphorylated peptides are not readily identifiable from unenriched samples due to their sub-stoichiometric appearance (e.g. [1]). Fractionation followed by enrichment is time consuming, expensive and requires specialized equipment and operators. Here we present a method which addresses these problems by reversing the workflow, performing first batch-enrichment of phosphopeptides followed by their fractionation using in house manufactured tip columns [2]. This allows for cost- and time-efficient identification of large quantities of phsophopeptides without specialized equipment.

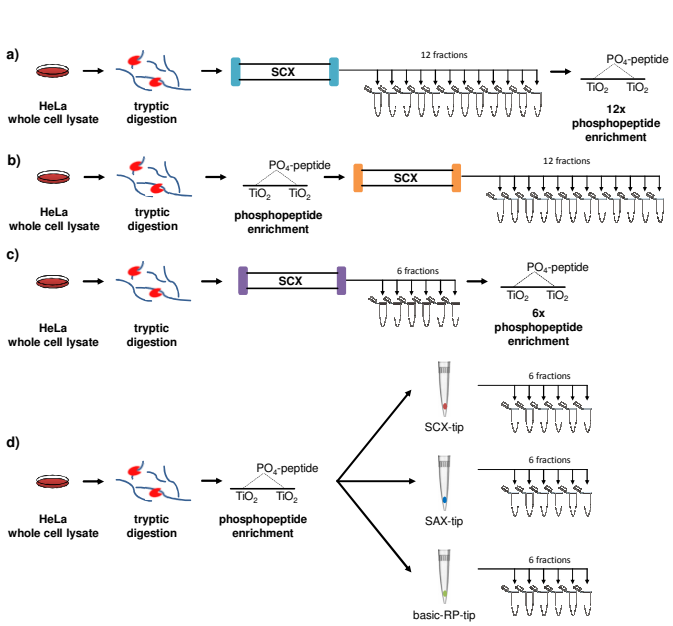

Figure 1: Strategies for the enrichment and fractionation of phoshopeptides from tryptic digests of HeLa whole cell lysate. Peptides were either fractionated by SCX chromatography followed by enrichment of phosphoeptpides from the single fractions (a, c) or enriched in batch mode followed by fractionation of the enriched peptides (b, d). For fractionation of enriched peptides either a HPLC system (a, b, c) or self made tip columns and a centrifuge (d) were used.

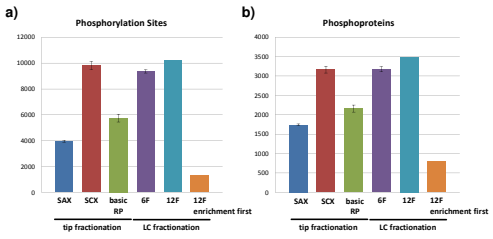

Figure 2: Comparison of the number of identified phosphorylation sites (a) and phosphoproteins (b) for the different approaches. F= number of fractions

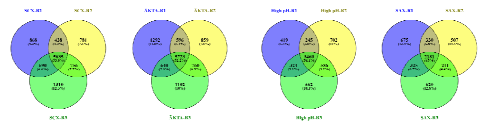

Figure 3: Reproducibility of phosphopeptide identification across replicates for different fractionation methods. Displayed are numbers of identified overlapping phosphopeptides.

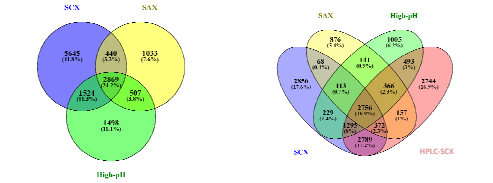

Figure 4: Orthogonality of phosphopeptide identification for the different fractionation methods. Displayed are numbers of identified overlapping phosphopeptides.

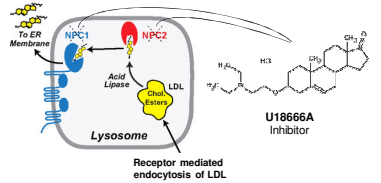

Figure 5: Mode of action for the cholesterol transport inhibitor U18666A. The compound prevents the transport of cholesterol-ester derived cholesterol from the lysosome to the rest of the cell (adapted from [4]).

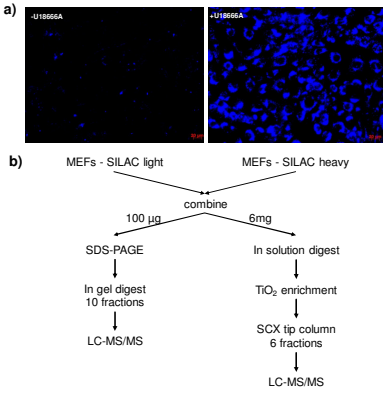

Figure 6: a) Staining of U18666A-treated and control cells with the cholesterol visualizing compound Filipin. b) Workflow for the quantitative proteomic analysis by stable isotope labeling of amino acids in cell culture (SILAC).

5503 proteins: ↑91 ↓31 | 12882 phospho sites: ↑ 349 ↓ 402

Protein	H/L
Squalene monooxygenase	9.0
Septin-9	8.9
Delta(14)-sterol reductase	8.0
Abhydrolase domain-containing protein 2	7.0
Squalene synthase	6.8
Lanosterol synthase	6.2
Sterol-4-alpha-carboxylate 3-dehydrogenase	5.8
Delta(24)-sterol reductase	5.8
Lanosterol 14-alpha demethylase	5.2
Methylsterol monooxygenase 1	4.9
3-keto-steroid reductase	4.8
7-dehydrocholesterol reductase	4.7
Acetyl-coenzyme A synthetase, cytoplasmic	4.2
Isopentenyl-diphosphate Delta-isomerase 1	4.2
Tapasin	3.8
Diphosphomevalonate decarboxylase	3.8
Retinol dehydrogenase 11	3.7
3-hydroxy-3-methylglutaryl-coenzyme A reductase	3.7
Acetyl-CoA acetyltransferase, cytosolic	3.6
Phosphomevalonate kinase	3.5

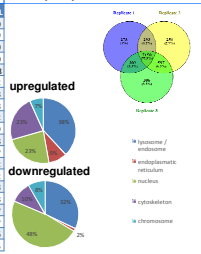

Figure 7: Results of quantitative proteomic and phosphoproteomic analyses of U18666A treated MEFs. Among the top 20 regulated proteins 18 are involved in cholesterol metabolism (bold underlined). 78% of phosphopeptides were quantified in 3 replicates. Go analysis of regulated phosphorylation sites.

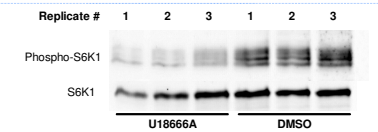

Figure 8: Confirmation of decrease of S6 kinase phosphorylation implying deactivation of mTOR upon treatment with U18666A.

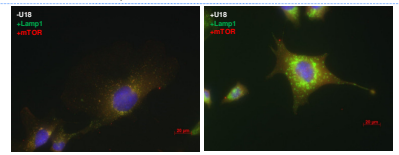

Figure 9: Co-staining of a lysosomal marker protein (Lamp1) and mTOR confirming deactivation of mTOR after U18666A treatment due to dissociation from the lysosome.

METHODS

For protocol establishment and optimization HeLa whole cell lysate was digested in solution and peptides desalted using Oasis Hlb cartriges. Peptide fractionation / phosphopeptide enrichment was then performed in different workflows: i) peptides were fractionated using strong cation exchange (SCX) chromatography with an HPLC system followed by titanium dioxide based enrichment of phosphopeptides for each of the resulting fractions; ii) phosphopeptides were enriched using TiO2 and fractionated using the HPLC system with SCX; iii) phosphopeptides were enriched by titanium dioxide and then fractionated by tip columns. Tip columns were manufactured in house using either strong anion exchange (SAX), SCX, or basic reversed phase disks. All samples were measured by LC-MSMS on an Orbitrap Velos mass spectrometer. Using an optimized method with SCX tip based columns, SILAC mouse embryonic fibroblasts (MEFs) were analyzed in a 2 plex experiment: Heavy labeled cells were treated with the cholesterol transport inhibitor U18666A and combined with SILAC light labeled untreated control cells. Phosphopeptide enrichment was performed using TiO_2 and enriched samples were fractionated using SCX tips. Single fractions were analyzed by LC-MSMS followed by peptide identification and quantification with Maxquant [3]. Candidate proteins were confirmed by Western Blot and microscopy.

RESULTS METHOD OPTIMIZATION

We prepared a large amount of HeLa whole cell lysate tryptic digest in order to have a reproducible source of peptides. For each strategy we used 3mg of starting material and performed 3 independent replicates except for the 12 fraction-HPLC-SCX experiments. For LC-SCX fractionation we observed reasonable results (10,216 sites from 12 fractions), only when fractionating peptides followed by enrichment for phosphopeptides. When we reversed the order performing enrichment before fractionation numbers of identified phosphopeptides decreased strongly to ~10% of the values obtained when fractionating first (1298). For tip-based methods, 6 fractions were generated. On average, SCX tips yielded the highest numbers of identified phosphosites (9812) compared to SAX (3950) and basic RP (5740). SCX tip based fractionation outperformed SCX column fractionation followed by enrichment of single fractions (9354) when the same number of fractions was generated (6 fractions) even though the SCX column samples were acquired with longer LCMS gradients (120 vs. 90min). All tip-based approaches identified certain subsets of phosphopeptides, whereas the overlap is biggest between the SCX and basicRP tips and the number of unique peptides is highest for the SCX tips. Reproducibility of number of identified phosphopeptides was excellent for all strategies .

RESULTS U18666A TREATMENT OF MEFS

The compound U18666A inhibits cholesterol egress from the lysosome resulting in its accumulation in lysosomes mimicking the phenotype of Niemann Pick Disease type C. Heavy SILAC labeled MEFs were treated with U18666A, combined with untreated light cells in 3 independent biological replicates and phoshopeptides enriched by TiO_2 followed by fractionation using SCX tips. In total, we identified and quantified 12881 phosphosites. 7150 phosphoeptides (representing 78% of the total number of identified phosphopeptides) were quantified in all 3 replicates demonstrating the excellent reproducibility of our enrichment / fractionation protocol. On the protein level, among the top 20 regulated proteins, we found alterations in major signaling pathways and performed Western Blot based follow up on selected proteins confirming the mass spectrometry data. Among others, mTOR signaling was altered which was confirmed by Western blot analysis of the mTOR target S6 kinase and by investigation of mTOR localization at the lysosome.

REFERENCES

[1] Villén J, Gygi SP. Nat Protoc. 2008; 3(10): 1630-8. [2] Rappsilber J. et al. Anal Chem. 2003 Feb 1; 75(3): 663-70.
[3] Cox J, Mann M. Nat Biotechnol. 2008 Dec; 26(12): 1367-72. [4] Infante R.E. et al. PNAS 2008; 105: 15287-15292.